

# The University of Bradford Institutional Repository

<http://bradscholars.brad.ac.uk>

This work is made available online in accordance with publisher policies. Please refer to the repository record for this item and our Policy Document available from the repository home page for further information.

To see the final version of this work please visit the publisher's website. Access to the published online version may require a subscription.

**Link to publisher's version:** <https://doi.org/10.1016/j.toxlet.2018.04.011>

**Citation:** Spriggs S, Cubberley R, Loadman P et al (2018) A study of inter-individual variability in the Phase II metabolism of xenobiotics in human skin. *Toxicology Letters*. 292: 63-72.

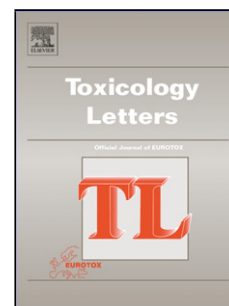
**Copyright statement:** © 2018 Elsevier B.V. Reproduced in accordance with the publisher's self-archiving policy. This manuscript version is made available under the [CC-BY-NC-ND 4.0 license](#).



## Accepted Manuscript

Title: A study of inter-individual variability in the Phase II metabolism of xenobiotics in human skin

Authors: Sandrine Spriggs, Richard Cubberley, Paul Loadman, David Sheffield, Antonia Wierzbicki



PII: S0378-4274(18)30146-2  
DOI: <https://doi.org/10.1016/j.toxlet.2018.04.011>  
Reference: TOXLET 10159

To appear in: *Toxicology Letters*

Received date: 26-10-2017  
Revised date: 2-3-2018  
Accepted date: 12-4-2018

Please cite this article as: Spriggs, Sandrine, Cubberley, Richard, Loadman, Paul, Sheffield, David, Wierzbicki, Antonia, A study of inter-individual variability in the Phase II metabolism of xenobiotics in human skin. *Toxicology Letters* <https://doi.org/10.1016/j.toxlet.2018.04.011>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title:

## A study of inter-individual variability in the Phase II metabolism of xenobiotics in human skin

Authors: Sandrine Spriggs <sup>1(\*)</sup>, Richard Cubberley <sup>1</sup>, Paul Loadman <sup>2</sup>, David Sheffield<sup>1</sup> and Antonia Wierzbicki <sup>2</sup>

Contact addresses:

1 Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire, MK44 1LQ.

2 Institute of Cancer Therapeutics, University of Bradford, Bradford, UK, BD7 1DP

(\*) Corresponding author: Tel: +44 1234 264743, e-mail address: sandrine.spriggs@unilever.com

Highlights

- Evaluated various handling and storage conditions post-surgery
- Developed method for preparation of S9 with emphasis on cofactor addition
- Activity of Phase II enzymes measured in up to 90 individuals
- Comprehensive statistical analysis of results

Abstract

Understanding skin metabolism is key to improve *in vitro* to *in vivo* extrapolations used to inform risk assessments of topically applied products. However, published literature is scarce and usually covers a limited and non-representative number of donors. We developed a protocol to handle and store *ex vivo* skin samples post-surgery and prepare skin S9 fractions to measure the metabolic activity of Phase II enzymes. Preincubation of an excess of cofactors at 37°C for fifteen minutes in the S9 before introduction

of the testing probe, greatly increased the stability of the enzymes. Using this standardised assay, the rates of sulphation (SULT) and glucuronidation (UGT) of 7-hydroxycoumarin, methylation (COMT) of dopamine and N-acetylation (NAT) of procainamide were measured in the ng/mg protein/h (converted to ng/cm<sup>2</sup>/h) range in eighty-seven individuals. Glutathione conjugation (GST) of 1-chloro-2,4-dinitrobenzene was assessed in a smaller pool of fifty donors; the metabolic rate was much faster and measured over six minutes using a different methodology to express rates in µg/mg protein/min (converted to µg/cm<sup>2</sup>/min). A comprehensive statistical analysis of these results was carried out, separating donors by age, gender and metabolic rate measured.

### Abbreviations

ACD: Allergic contact dermatitis. COMT: catechol-O-methyl transferases. GST: glutathione-S-transferases. HaCaT: Immortalised keratinocytes “Human Addult Low Calcium High Temperature”. NAT: N-acetyltransferases. NHEK: Normal Human Epidermal Keratinocytes. SULT: sulphotransferases. UGT: UDP-glucuronyltransferases.

### Keywords

Phase II metabolism, *ex vivo* skin, S9 incubations, cofactors

### Introduction

Human skin forms the first protective barrier between our body and the environment (Bouwstra and Ponc, 2006; Madison, 2003). Despite being accepted as a physical barrier, the skin is under constant exposure to xenobiotic chemicals able to diffuse through the skin layers (Berard et al., 2003; Zalko et al., 2011). This could potentially cause toxicity in the skin itself and other parts of the body due to systemic exposure. The second line of defence in the skin’s barrier function, often described as a chemical barrier,

is its capacity to detoxify and eliminate these compounds via metabolism using phase I and phase II metabolic enzymes (Oesch et al., 2014; Svensson, 2009). Although cutaneous metabolism is actively exploited in the application of dermally applied pro-drugs (Møllgaard et al., 1982), metabolic activation is almost certainly why seemingly unreactive compounds cause skin sensitisation reactions (Schmidt and Khan, 1989). Conversely, it can be argued that the sensitisation risk from a reactive chemical would be reduced if it were metabolised to a non-reactive form in the skin (Manwaring et al., 2015). The redox mechanisms present in the skin might also be involved (Korkina, 2016), though the two phenomena can be difficult to separate in cases such as glutathione metabolism. Thus, skin metabolism needs to be characterised thoroughly to understand its impact on the magnitude of adverse outcomes such as allergic contact dermatitis (ACD).

Published literature regarding cutaneous metabolism is limited and clearance mechanisms within skin are still poorly understood. Studies on the mRNA expression levels in skin have identified the presence of phase I and phase II enzymes, including the phase II enzymes UDP-glucuronyltransferases (UGT), sulphotransferases (SULT), N-acetyltransferases (NAT), catechol-O-methyl transferases (COMT) and glutathione-S-transferases (GST) (Hu et al., 2010; Luu-The et al., 2009; van Eijl et al., 2012). However, mRNA levels often show poor correlation to protein expression levels (Maier et al., 2009) and quantitative measurement of metabolism in skin remains a challenge.

The closest experimental approach under which to study metabolism in skin to compare to *in vivo* situations, is to use viable *ex vivo* human skin in culture (Manevski et al., 2015; Zalko et al., 2011). Effectively a living system, the various compartments (both inter and intra cellular) remain relatively intact and tissue viability as well as the levels of some biomarkers are preserved for several days (de Wever et al., 2015; Varani et al., 2007). Several studies have used the incubation of whole skin in media containing concentrations of chemical substrate to study activity rates (Eilstein et al., 2014; Manevski et al., 2015), while topical application of the substrate on cultured skin explants or models is favoured in studies covering both skin penetration of the test substrate and identification of its major metabolites (Jacques

et al., 2014). Whilst such studies have shown promising results, obtaining fresh *ex vivo* skin consistently is difficult, and these studies often represent only a small number of donors.

Models commonly used in metabolism studies include primary keratinocytes such as Normal Human Epidermal Keratinocytes (NHEK) or immortalised keratinocytes such as the “Human adult Low Calcium High Temperature” (HaCaT) cell line. The N-acetylated product of 4-amino-2-hydroxytoluene, a common hair dye, has been reported in both *ex vivo* skin and HaCaT experiments (Goebel et al., 2009). The activity level of NAT1 enzyme in HaCaT cells has been estimated to be 3.4 fold higher than in NHEK (Bonifas et al., 2010) but still comparable to levels expected in the skin. Models more closely related to skin such as reconstructed 3D skin models consisting of keratinocytes-derived layers (e.g. EpiSkin, EpiDerm, Phenion) and their Full Thickness versions, which include a layer of cultured fibroblasts in collagen under the keratinocytes to mimic the effect of the dermis, have been used, but understanding the differences between all these models and *ex vivo* skin is still the subject of investigations (Eilstein et al., 2014; Gotz et al., 2012a; Gotz et al., 2012b; Hewitt et al., 2013; Jackh et al., 2011; Wiegand et al., 2014).

When skin culture is not practical, freshly excised skin can be preserved, typically by snap freezing in liquid nitrogen, and stored frozen. However, metabolism studies using frozen skin have yielded mixed results in the past and protocols tend to vary from study to study. We investigated the influence of storage on both fresh skin and S9 fractions and developed approaches to homogenise skin and handle S9 during metabolic assays. We used 7-hydroxycoumarin (7-HC) to study sulphation and glucuronidation (Wang et al., 2005), procainamide for acetylation (Dreyfuss et al., 1972), dopamine for methylation (Mannisto and Kaakkola, 1999) and 1-chloro-2,4-dinitrobenzene (DNCB) for glutathione conjugation (Harris et al., 2002). A standard protocol derived from these experiments was then applied to samples from up to 90 volunteers to generate metabolic rates for each of the five Phase II pathways studied. The metabolic rates obtained for each enzyme were statistically analysed to evaluate similarities within the dataset, mainly by comparing age or gender and creating “high metaboliser” versus “low metaboliser” groups based on one enzyme and comparing with the others. Even without consideration of statistical differences between

groups of individuals, the mean metabolic rate for each enzyme can still be a useful piece of information to integrate into *in silico* models of human skin.

*In silico* models aiming to extrapolate *in vitro* data to *in vivo* exposure scenarios are built using chemicals for which historical data is available such as caffeine (Gajewska et al., 2015) or para-phenylenediamine (Manwaring et al., 2015). While some examples have been provided to show that skin metabolism can be a major factor affecting skin bioavailability (Jacques et al., 2010; Jacques et al., 2014), refinements of these *in silico* models will require a broader use of skin detoxification potential data such as the ones presented here.

## Materials and methods

### **Chemicals and reagents**

Dopamine, procainamide, 7-hydroxycoumarin, 1-chloro-2,4-dinitrobenzene, S-adenosyl-methionine (SAM), 3'-Phosphoadenosine-5'-phosphosulphate (PAPS), Acetyl Coenzyme A (AcCoA), Uridine diphosphoglucuronic acid (UDPGA), glutathione (GSH), glutathione disulphide (GSSG), 7-hydroxycoumarin glucuronide (7-HCG), 7-hydroxycoumarin sulphate potassium salt (7-HCS), N-acetylprocainamide hydrochloride (NAPA) were purchased from Sigma Aldrich (Gillingham, UK) and used without further purification. The methylated dopamine standard, i.e. 3-methoxytyramine hydrochloride (3-MT), was purchased from Fisher Scientific Ltd (Loughborough, UK). Deuterated 7-hydroxycoumarin (7HC-d<sub>5</sub>) was purchased from BD BioSciences Ltd. All solvents were purchased at the highest purity available (HPLC grade minimum).

A mass labelled internal standard of the glucuronide of 7-HC, i.e. 7HC-d<sub>5</sub>-glucuronide (7-HCG-d<sub>5</sub>) was created in house by incubating 200µg/mL 7HC-d<sub>5</sub> in concentrated skin S9 containing 5mM UDPGA for 4 hours at 37°C. 7-HCG was isolated from a methanol extract by HPLC and stored at -80°C until use.

Dinitrophenyl-glutathione (DNP-SG), the GSH conjugate formed with DNCB, was created in house by incubating 200µg/mL DNCB in concentrated skin S9 for 4 hours at 37°C. The mixture was extracted in acetonitrile, the supernatant separated by HPLC, the fraction containing the metabolite DNP-SG obtained frozen at -20°C until use.

### ***Human skin samples***

All skin samples were sourced from the Bradford Ethical Tissue Bank. The Ethical Tissue Bank was authorized by the Leeds flagged REC to release samples to researchers. The Ethical Tissue Bank also acts on behalf of the Recipient's Institution for the collection, use and storage of Material and associated data. Samples were provided anonymously with only the minimum data set and handled in compliance with the Human Tissue Act. All full thickness skin samples were frozen within 120 minutes of excision.

### ***Protocol optimisation for skin S9 preparation***

First, freshly excised skin samples were cut to size (triangle of approximately 1cm side length), weighed and measured (the height and base of each triangle was measured with a ruler and the surface area calculated), homogenised in Phosphate Buffered Saline (PBS) (4mL/g skin) on ice using an Ultra Turrax blender (IKA) (4°C, 30 seconds). The homogenate was then centrifuged at 9000g (4°C, 20 minutes) and the S9 fraction collected as the supernatant.

As a separate experiment, freshly excised skin triangles were snap frozen in liquid nitrogen and pulverised using a cell crusher (Stratech) using a mallet until the frozen skin had a dust-like powder appearance. PBS was added to the pulverised tissue (4mL/g skin) and the sample sonicated on ice (4°C, 6x30 seconds).

The protein content of the resulting S9 was determined using the Bradford Method (Bradford, 1976). We compared the two methods of homogenisation for a single skin sample by submitting fractions of S9 generated by each approach to the same incubations with 7-HC, dopamine and procainamide (with corresponding cofactor).

### ***Protocol optimisation for preserving enzymatic stability***



First, to assess the effect of time on the handling procedure for fresh *ex vivo* skin (post-surgical procedure), whole skin (one triangle placed in PBS, volume adjusted to the weight of each triangle so that the ratio was 4mL/g tissue) and freshly prepared S9 fraction (skin homogenised in PBS at a concentration of 4mL PBS per g of tissue) from the same single donor were incubated at 37°C or 25°C for 0, 3, 6 and 24 hours, after which the incubated skin was homogenised to prepare S9 for analysis (as above) and the incubated S9 was used as collected. Enzymatic activity was assessed at each time point for all enzymes except GST. The rates were measured in ng metabolite/mg protein/h, the initial rate fixed at 100% activity and all subsequent activities expressed as a percentage depletion of the initial rate.

GST activity is thought to be mostly dependant on the availability of its cofactor, GSH, which is prone to oxidation. To determine the effect of freeze-thawing on GSH levels, whole skin samples from five donors were aliquoted. Half the samples were analysed immediately for GSH and GSSG content (qualitative measurement expressed in peak area), while the other half were frozen and stored at -80°C then thawed the following day and analysed. GST activity was assessed in whole skin from a single donor by monitoring the formation of DNP-SG in freshly prepared S9 and S9 prepared after one freeze thaw cycle (-80°C for one day), with and without addition of GSH.

Finally, the time at which cofactor addition was carried out in S9 incubations was investigated. Using S9 prepared from a single donor, cofactors were either introduced immediately upon preparation of the S9 and the S9 fractions then pre-incubated for 15 minutes before addition of the probe, or the cofactors were added to the S9 at the same time as the probe substrate. Both sets of samples were then incubated for 0, 10, 30, 60 and 190 minutes. The activity of GST being significantly faster than other enzymes studied, these pre-incubation studies were not carried out. Instead, an arbitrary high concentration of GSH (10µg/mL) was added immediately after preparation of S9 fractions.

#### ***Skin S9 preparation (optimised method)***

Skin samples were either snap frozen in liquid nitrogen and stored whole at -80°C until use or used fresh within 120 minutes of collection from the clinic. Freshly defrosted skin samples or freshly excised skin

samples were cut to size (triangle with 1cm base), weighed and measured, homogenised in PBS (4mL/g skin for standard enzymatic assay, 50mL/g skin for GST activity) on ice using an Ultra Turrax blender (IKA) (4°C, 30 seconds). The homogenate was then centrifuged at 9000g (4°C, 20 minutes) and the S9 fraction collected as the supernatant.

#### ***Skin S9 incubations (optimised method)***

Each S9 fraction was split into multiple aliquots to study glucuronidation, sulphation, methylation, acetylation and glutathione conjugation separately. S9 aliquots were pre-incubated for 15 minutes at 37°C with specific cofactors in each tube, SAM (2.5mM), PAPS (5mM), AcCoA (1mM), UDPGA (0.5mM) or GSH (10 µg/mL). Dopamine, procainamide, 7-HC were then introduced at 2µg/mL and DNCB at 10µg/mL to the S9.

At each time point (t= 0, 1, 2 and 4 hours for UGT, SULT, COMT and NAT activities, t= 0, 3 and 6 minutes for GST activity), one volume of the metabolism assay mixture was removed and diluted with three volumes of ice cold methanol (containing the internal standard 7-HCG-d<sub>5</sub> when required or 0.2% formic acid for the GST assay samples) and the mixture centrifuged at 10 000g for 4min (4°C). Supernatants were dried down using a rotary evaporator and reconstituted in water: methanol (90:10 v/v).

#### ***Assessment of skin metabolic activity in multiple donors***

Over a period of two years, 87 donor skin samples were collected and analysed for metabolic activity of glucuronidation, sulphation, acetylation and methylation. The population was 83% female and 17% male, aged between 17 and 90 years old. Age was not specified in 4 out of the 72 samples given by women. Calculations were therefore carried out on a set of 68 samples. The mean age for women donors was 45.9± 9.6 years (the median age being 46.5 years old). The youngest female donor was 20 years old and the oldest 65 years old. Similar calculations were carried out with male samples when information about age was provided (15 out of 17). The mean age for men donors was 51.1± 17.8 years (the median age being 54 years old). The youngest male donor was 17 years old and the oldest 90 years old. The majority of skin samples were taken from the abdomen, with some taken from other areas such as arm, breast and

thigh. Afterwards, 50 samples were assayed to measure glutathione conjugation rate, 48 of which being from the same donors as the previous analysis, picked randomly, as the amount of frozen skin was still available. The population for this analysis was 82% female and 18% male, aged between 20 and 90 years old, with most donors being in the range 40-60 years old.

The rate of metabolism was expressed as ng/mg protein/hour in the standard assay for the formation of 7-HCG, 7-HCS, NAPA and 3-MT. For statistical analysis and ease of understanding (doses used in skin penetration data are usually expressed per cm<sup>2</sup>) these were also converted to ng/cm<sup>2</sup>/h: for each sample, the skin triangle was measured so that the surface area could be calculated (triangles were cut and their base and height was measured with a ruler to calculate the surface area) and weighed so that results could be expressed per mg tissue if required. The volume of PBS used for generating the S9 was 4mL/g tissue. The S9 protein content (as determined by Bradford method) was expressed in mg protein/mL.

Converted rate ( $\mu\text{g}/\text{cm}^2/\text{h}$ ) = [Rate ( $\mu\text{g}/\text{mg protein}/\text{h}$ ) x Protein concentration (mg/mL)] / [Volume PBS in S9 (mL) x Surface area of skin triangle (cm<sup>2</sup>)]

Due to the speed at which GST activity was measured, i.e. the amount of DNP-SG formed at the initial time point was already significant, the rate of metabolism was presented as the slope of the linear regression, expressed in  $\mu\text{g}/\text{mg protein}/\text{min}$ , and the intercept at the origin, expressed in  $\mu\text{g}/\text{mg protein}$ . For ease of understanding and statistical analysis this was converted to  $\mu\text{g}/\text{cm}^2/\text{min}$  following the same method as the one used for the other enzymes.

All rates measured are presented per enzyme in Supplementary data, Figures D-H.

#### ***LC-MS-MS analysis***

Metabolite quantification was determined by LC-MS analysis of sample extracts using calibration standards containing the expected metabolites (with the addition of the internal standard 7-HCG-d<sub>5</sub> for 7-HC standards).

An Acquity Ultra-High Pressure Liquid Chromatography (UPLC) system from Waters was used for the LC separation of the compounds of interest. Chromatographic separation was obtained using an Acquity UPLC BEH C18 column from Waters (1.7 $\mu$ m, 2.1x100mm for standard enzymatic assay, 1.7 $\mu$ m, 2.1x150 mm for GST assay) with a column temperature of 40°C. Mobile phases consisted of 1% methanol in distilled water containing 0.1% formic acid (mobile phase A) and 90% Methanol in distilled water containing 0.1% formic acid (mobile phase B). A flow rate of 0.3mL/min was used. In the standard assay, the mobile phase composition was initially set to 100% A, then increased to 20%B over 20 minutes, the gradient was then increased to 100% B for 2 minutes, before re-equilibrating to 100% A for the remaining 8 minutes. In the GST assay, the mobile phase composition was initially set to 100% A for 5 minutes, then increased to 100% B over 10 minutes, the gradient was held 100% B for 5 minutes, before re-equilibrating to 100% A for the remaining 10 minutes.

A Quattro Premier Mass Spectrometer (MS) from Waters was used with an ESI (Electrospray Ionisation) source. All samples were analysed using Multiple Reaction Monitoring (MRM) for the complete length of the UPLC acquisition time (30min). In positive ion mode, the MRM transitions used were: for 7-HCG m/z 339.93>162.86 (Collision energy 25eV), for 7-HCG-d<sub>5</sub> m/z 344.27>167.86 (25eV), for 3-MT m/z 167.8>151.3 (12eV), for NAPA m/z 278.07>205.05 (25eV) and for DNP-SG m/z 475.40>345.30 (25eV). The ESI source was operated in negative ionisation mode for the detection of 7-hydroxycoumarin sulphate (7HCS) with a MRM transition m/z 241.3>160.8 (15eV).

Data processing was carried out using MassLynx software (Version 4.1), and concentrations of metabolites formed at each time point were calculated from standard curves.

#### ***Statistical analysis and correlations between groups of the population studied***

Statistical analysis of the results was carried out using the SAS version 9.4 software (SAS Institute, Marlow, UK) and pictures produced using the software. Results from one donor (2372) were removed from the statistical analysis as rates measured were negative for two enzymes and very close to zero for the other enzymes, hinting at a loss of metabolic activity linked to sample handling rather than a donor deprived from metabolic capacity for all enzymes. The sample from donor 2970 was used for GST analysis. All

instances of negative values (three occurrences in N-acetylation) were artificially fixed to zero. Reaction rates were converted to ng/cm<sup>2</sup>/h (UGT, NAT, COMT, SULT) and µg/cm<sup>2</sup>/min (GST). However, a square root transformation was applied to ensure that the largest values do not have excessive influence over the statistical analysis.

We investigated potential correlations between metabolic activity and the gender of the skin donors by fitting a linear regression  $y = a + b(\text{gender})$  where gender equals 1 for males or 0 for females to the data and testing the effect of  $b$  (P-value). The test was carried out using the GLM (General Linear Model) procedure in SAS.

Evaluation of correlations between metabolic activity and the age of the skin donors was carried out by fitting a linear regression with age as the only factor. If the gradient of the slope of the line was significantly greater than zero, age was deemed a significant factor. A P-value was obtained to estimate the difference between the gradient and zero. Scatter plots representing square root of enzymatic activity versus age were constructed using the REG (Regression) Procedure in SAS.

We also questioned whether a donor showing a high metabolism rate for one enzyme (i.e. SULT) would demonstrate a high level for all enzymes, helping to define a person as a “high metaboliser”. Or conversely, would a person with a low activity for one enzyme would also have low metabolic activity for all enzymes. These analyses covered all enzymes studied excepting GST. We plotted the square root of one enzyme versus another for each donor and highlighted the metabolisers that had high activity (top 25% of recorded values) for all enzymes or a low activity (bottom 25% of recorded values) for all enzymes for illustrative purposes.

## Results

***The use of a mechanical homogeniser for skin S9 preparation is preferable for metabolic rates measurements***

To investigate homogenisation methods limiting temperature increases while delivering consistent enzyme recovery, we compared mechanical homogenisation on ice using the Ultra Turrax homogeniser to a “cell crusher” method, which required snap frozen tissue to be pulverised under force. The use of the cell crusher, whilst reducing risks posed by heat generation of mechanical techniques, was found to leave clumps of tissue and ultrasonication was required to fully extract enzymes, which caused sample heating. Both sets of S9 generated were submitted to the same incubations with 7-HC, dopamine and procainamide (with corresponding cofactor) and led to comparable metabolic rates (Table 1), except for glucuronidation which was ten times faster in Ultra Turrax prepared samples.

Enzyme	Metabolic rate “Cell crusher”	Metabolic rate “Ultra Turrax”
UGT	1	10
SULT	94	98
COMT	14	20
NAT	3	2

Table 1: **Metabolic rates in S9 fractions prepared by manual pulverisation or mechanic homogenisation are comparable.** Both sets of S9 were prepared from a single donor and used immediately with 7-HC, dopamine and procainamide. Metabolic rates are expressed in ng metabolite/mg protein/h (n=1).

For practical reasons and considering the improvement observed for UGT activity, all subsequent S9 samples were prepared using the Ultra Turrax homogeniser method.

***Fresh ex vivo skin samples should be snap frozen immediately, whole, in liquid nitrogen or used fresh within 2 hours***

Maintaining whole skin samples or S9 fractions at either 25°C or 37°C, over 24 hours, reduced enzymatic activity in whole skin and S9 significantly for all four enzymes in our standard assay (Table 2). Metabolic rates in whole skin were better preserved when the skin was maintained at 37°C rather than 25°C, for all enzymes except NAT. However, the reverse effect was observed for S9 fractions, which had lost tissue integrity during preparation and were more stable at 25°C. After 3 hours, the enzymatic activity was reduced by 10-38% in whole skin samples kept at 37°C and 2-78% in S9 fractions kept at 25°C. We would

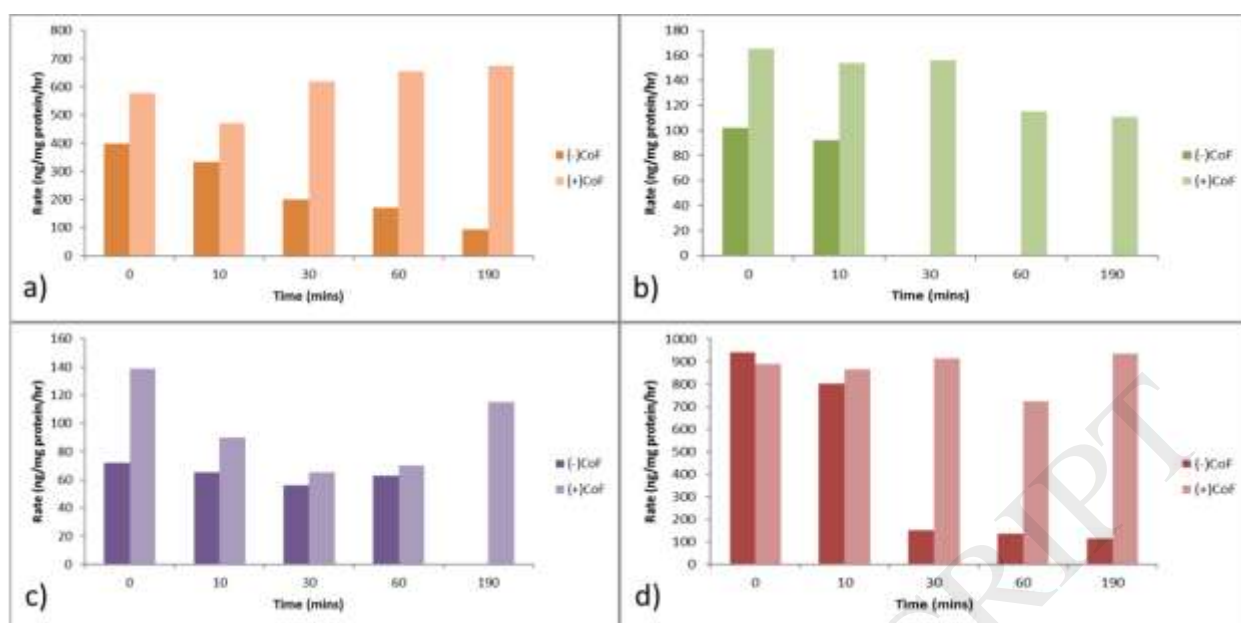
therefore recommend using *ex vivo* skin within 2 hours post-surgery to carry out metabolism studies. In a separate experiment, GST activity was significantly lower following freeze thawing when no additional cofactor was added. However, the addition of GSH was found to restore activity to pre-freezing levels (Supplementary data, Figure C).

Temperature ( in °C)	Whole skin						S9 fraction					
	25			37			25			37		
Time points (in hours)	3	6	24	3	6	24	3	6	24	3	6	24
UGT	49	70	62	33	44	60	5	21	50	22	54	50
SULT	30	60	100	10	42	100	30	26	40	42	50	68
COMT	59	73	42	22	19	30	2	23	85	79	82	100
NAT	25	39	27	38	59	83	78	72	93	100	100	102

Table 2: Enzymatic activity in whole skin samples or S9 fractions for UGT, SULT, COMT and NAT after storage at 25°C or 37°C for up to 24 hours. Enzymatic activity is expressed as a percentage reduction compared to the enzymatic activity measured at t= 0 (start of the experiment). (n=1).

#### ***Pre-incubation of S9 fractions with cofactors for enzymatic activities***

We investigated the influence of time at which the cofactors were first put in contact with Phase II enzymes. We found that the addition of cofactor immediately on preparation of the S9 fractions, followed by 15 minutes pre-incubation at 37°C before the inclusion of the chemical probe tested, might be beneficial for enzymatic activity measurements (Figure 1). For this particular skin sample, the most significant effect was found with the addition of AcCoA, where NAT activity would otherwise be completely lost within 10 minutes of incubation (Figure 1b). Conversely, pre-incubation with UDPGA showed little improvement to UGT activity during the first hour and the benefit from the technique was only visible at the 190 minutes time point (Figure 1c). Overall, we would recommend that all S9 fractions were pre-incubated with the cofactors (separately) for 15 minutes before the addition of the chemicals tested.



**Figure 1: Illustrating the effect of cofactor addition on enzymatic activity in skin S9 incubated at 37°C for 190 minutes.** Enzymatic activity in skin S9 for a) sulphation, b) N-acetylation, c) glucuronidation and d) O-methylation where (- CoF) S9 fractions were used with cofactors added at the same time as the chemical tested (*dark colour*) and (+ CoF) S9 fractions were pre-incubated with cofactors for 15min before addition of the chemical tested (*light colour*) (n=1).

#### **Metabolic activity measurements in multiple donors**

All donors showed metabolic activity for one or more enzymes, with the majority showing measurable activity for all five enzymes. Details of all rates measured, per enzyme, are available in Supplementary data, Figures C-G. The mean metabolic rates measured for all enzymes are presented in Table 3. The mean rate of DNP-SG formation (GSH conjugate of DNCB) was very fast and a 1:50 dilution of skin S9 (in PBS, before addition of GSH) was used in incubations to reduce the rate of the reaction, which was then measured over 6 minutes. The initial amount of DNP-SG measurable at t= 0min ranged from 0.00-5.19 $\mu$ g/mg protein, which was considered significant. Therefore, GST activity was reported as the slope of the linear regression (metabolic rate) and the intercept at the origin (Supplementary data).

Enzyme	Metabolic rate (ng/mg protein/h)	Metabolic rate (ng/cm <sup>2</sup> /h)
SULT	12.22 $\pm$ 14.82	262.70 $\pm$ 373.57
UGT	4.92 $\pm$ 6.39	109.94 $\pm$ 191.83
COMT	2.27 $\pm$ 1.94	47.03 $\pm$ 49.54
NAT	0.20 $\pm$ 0.20	4.37 $\pm$ 5.42
Enzyme	Metabolic rate ( $\mu$ g/mg protein/min)	Metabolic rate ( $\mu$ g/cm <sup>2</sup> /min)
GST	0.19 $\pm$ 0.22	0.99 $\pm$ 1.06

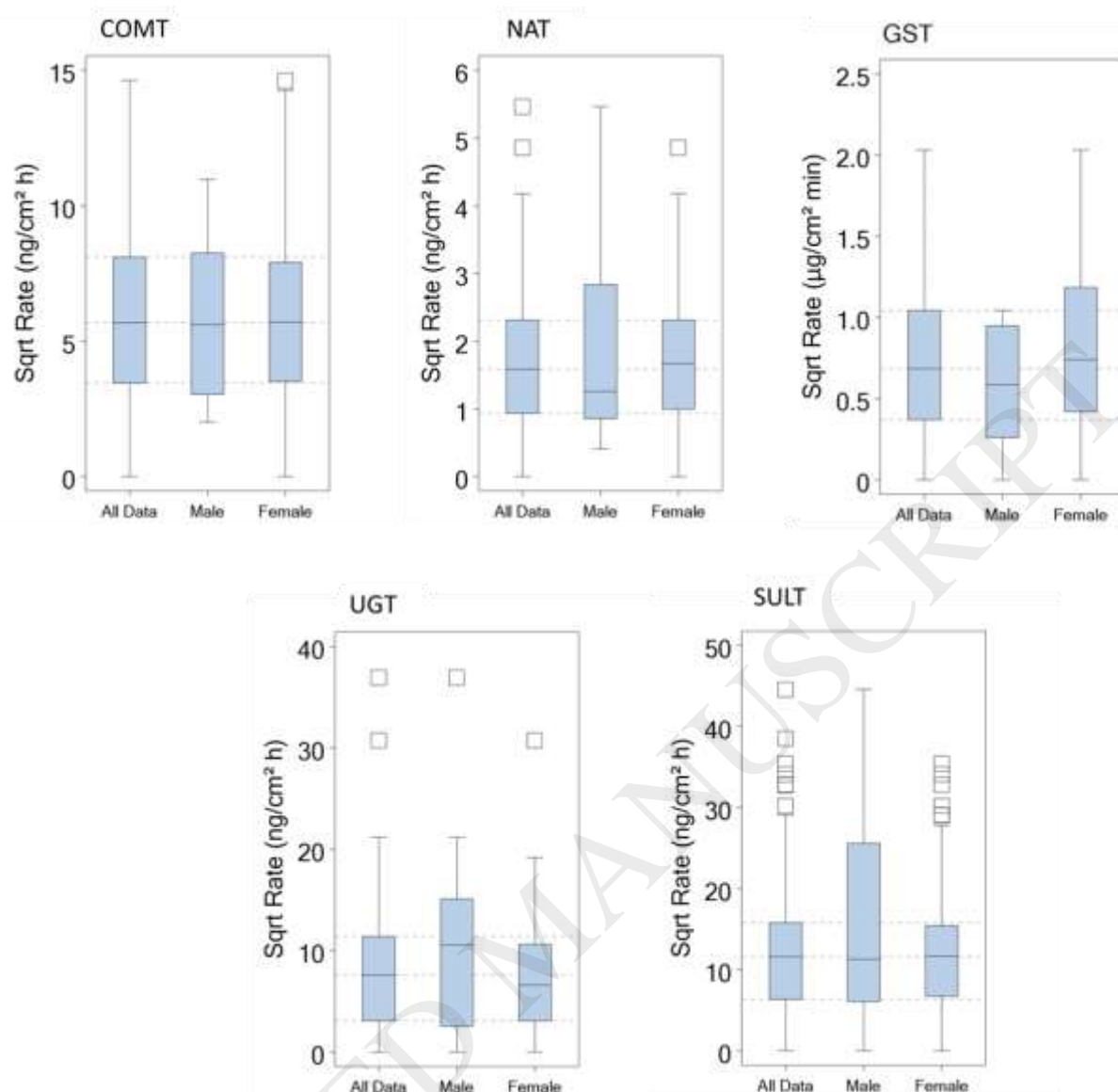


Table 3: **Metabolic rates measured in S9 fractions.** S9 fractions were incubated with 7-hydroxycoumarin, dopamine and procainamide for up to 4 hours and metabolic rates expressed in ng metabolite/mg protein/h (n=87). 1-chloro-2,4-dinitrobenzene was incubated for up to 6 minutes and the GST rate expressed in  $\mu\text{g}/\text{mg}$  protein/min (n=48).

For all enzymes, a high level of inter-patient variability was observed. Some of the variability might be imparted to differences in handling of skin post-surgery, whether used fresh within 120 minutes or snap frozen. High variability might also have originated from storage conditions and affected the five enzymes studies differently. Moreover, five samples did not show any GST activity and were reported as zero value (Supplementary data Figure D) but this has more likely been due to sample handling rather than being a true representation of GST activity for these donors.

***Metabolic activity measured in skin of donors is not gender related***

We investigated whether there was a correlation between gender and metabolic activity and carried out a linear regression. The P-values, ranging from 0.08 to 0.80, did not present enough evidence to relate gender and metabolic activity in our 90-donor study. A set of box plots representing the enzymatic activities measured for all donors and the subsets for each gender illustrates the lack of differentiation between male and female donors as both groups largely overlap with the overall activities measured (Figure 2).

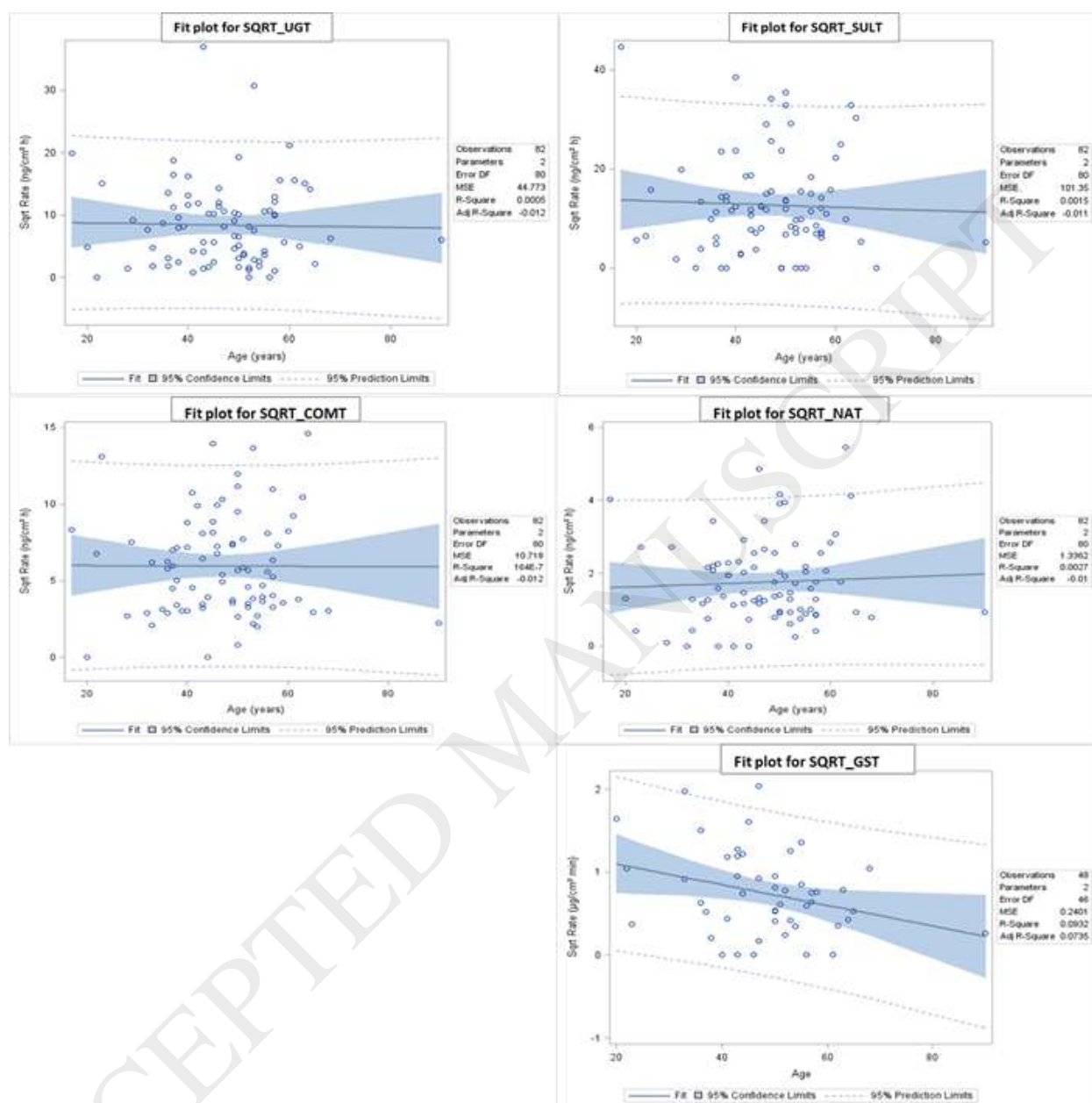


**Figure 2: Gender is not a differentiating factor for metabolic activity in skin.** Enzymatic activity in skin S9 for all enzymes (represented as Square root, expressed in ng/cm<sup>2</sup>/h or µg/cm<sup>2</sup>/min) where male and female donors have been compiled into two subsets and compared to the overall activity measured. Outliers are the values that are larger than 1.5 times the interquartile range above the 75<sup>th</sup> percentile (white squares) automatically selected by the software. (n =15 males, 71 females for COMT, NAT, UGT and SULT , 9 males, 40 females for GST)

### ***GST activity is age dependent***

We carried out a linear regression test on all five enzymes to establish whether enzymatic activity was linked to the age of the donor. We found that the GST activity level was negatively correlated to age (Pearson correlation statistic value: -0.3053, P-value 0.0348) but the other enzymes showed no evidence of correlation. To illustrate this further, we plotted the square root of the enzymatic activity (in ng/cm<sup>2</sup>/h

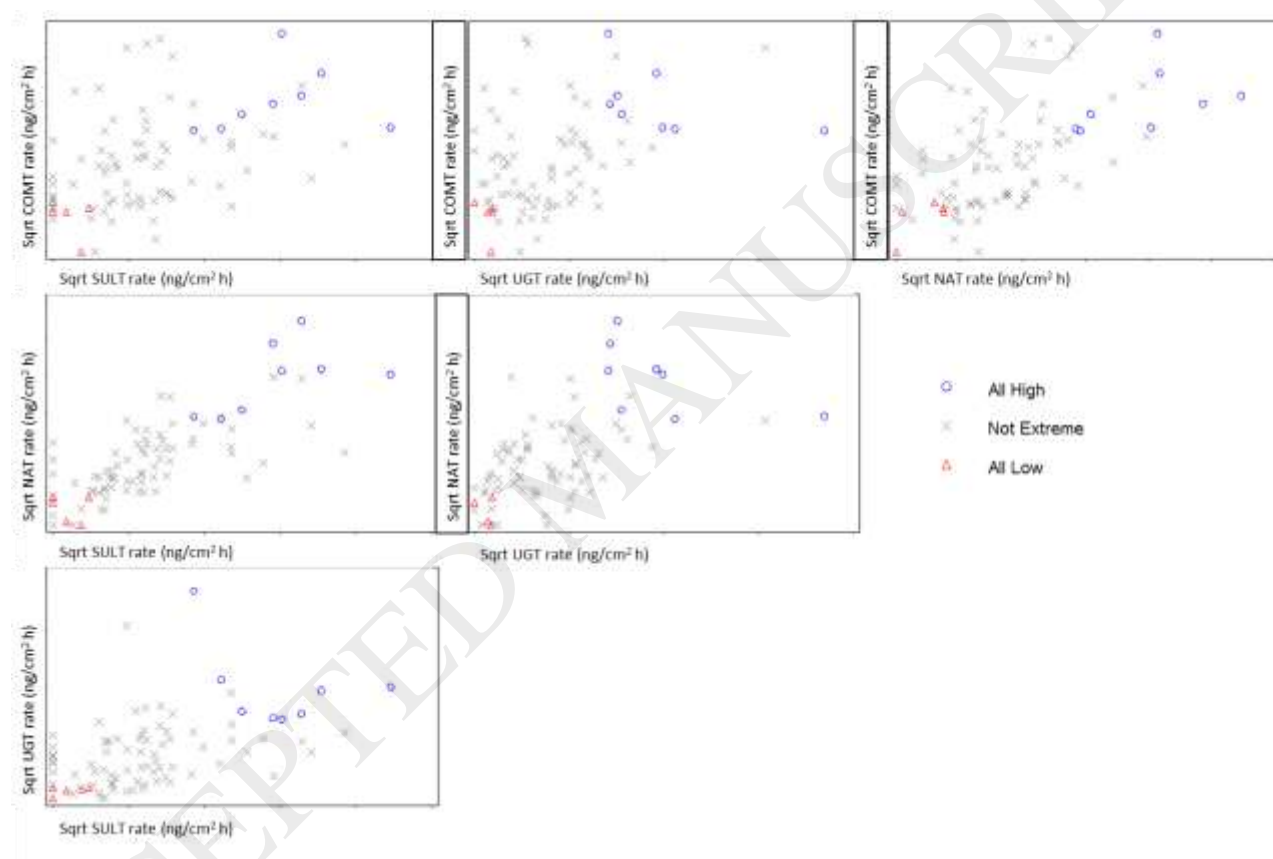
or  $\mu\text{g}/\text{cm}^2/\text{min}$  for GST) against the age of the donor (known for 82 out of 90 donors for NAT, COMT, UGT and SULT or 48 out of 50 donors for GST) (Figure 3).



**Figure 3: Correlation between enzymatic activity and the age of the donor.** Enzymatic activity was expressed in  $\text{ng}/\text{cm}^2/\text{h}$  or  $\mu\text{g}/\text{cm}^2/\text{min}$ . This is an overlay of the scattered data with a linear regression where values within the 95% confidence limit of the mean (filled blue) and 95% prediction interval of the data (blue spotted line) are estimated using the FITPLOT function. GST activity was negatively correlated to age but no relation could be established for the other enzymes. ( $n=82$  for UGT, COMT, NAT and SULT and  $n=48$  for GST).

#### Enzymatic activity level in a 90-donor study

It was found that most donors have a unique metabolic activity profile and may have a high activity for one enzyme and much lower than average activity for another (Figure 4). In a 90-donor study where statistical perfect order would be observed the subset of donors who would have high enzymatic activities for all four enzymes would represent 22 people, while the probability of a donor to have all enzymatic activities in the bottom 25% of recorded data by chance only is very low (0.4%), hence less than one donor would fit the criteria. Of the 87 donors analysed, 5 qualified as “low metabolisers” and 8 as “high metabolisers” (Figure 4).



**Figure 4: Enzymatic activity in a 90-donor study present very few cases of donors that could be identified as high or low metabolisers.** The square root of the activity of one enzyme is plotted against another. All permutations are presented, where donors who have enzymatic activity in the top 25% of recorded values for all enzymes (*blue circle*) and donors who are systematically in the bottom 25% (*red triangles*) have been differentiated from the pool of donors (*grey crosses*).

## Discussion

Understanding metabolism is key to successful *in vitro* to *in vivo* extrapolation in PBPK modelling. The identity and amount of chemical reaching different target sites is what ultimately leads to physiological effects and toxicity. Currently there is great interest in building more accurate models for cutaneous exposure, particularly for cosmetics ingredients. The impact of metabolism on local effects in the skin, such as sensitisation and genotoxicity is also of interest to toxicological risk assessors. There is no direct measure of skin metabolism in clinical or animal studies as metabolite levels in blood or urine are the result of metabolism from multiple tissue types. Similar *in vitro* approaches to that used to predict liver metabolism have been developed for skin, though the challenge of *in vitro* to *in vivo* extrapolation is arguably greater for skin due to the absence of specific skin metabolism data *in vivo*. Just as for liver metabolism, *in vitro* assays for skin metabolism need to ensure enzyme activity is preserved and appropriate conditions (such as cofactor levels) are used so that *in vitro* to *in vivo* extrapolation is meaningful.

Technically, preparing skin extracts can be difficult compared to softer tissues such as liver or kidney with the specific requirement for mechanical homogenisation which may accelerate enzyme deactivation (Berglund et al., 2007). Due to the complexity and potential instability of the enzymes in skin, a detailed knowledge of appropriate handling procedures is essential to producing reliable results in future skin studies. While the viability of skin is often described exclusively by the level of mitochondrial activity (MTT assay) still present in *ex vivo* skin purposefully preserved for surgical procedures (Alotto et al., 2002), little is known of the metabolic activity linked to clearance or defences against oxidative stress, though a few reviews are available (Gibbs et al., 2007; Oesch et al., 2014). Methods for *ex vivo* skin storage are limited: samples are either stored on culture medium (Lebonvallet et al., 2010) at 4°C, 37°C or cryopreserved using DMSO as a stabiliser. Samples that are not processed immediately post-surgery can see their viability levels decrease by a quarter over 30 hours and half within 60 hours (Castagnoli et al., 2003). We found that metabolic activity linked to the Phase II enzymes studied here, decreased even quicker (3-6 hours). We have established that enzymatic activity deteriorates in skin the moment it is removed from a donor and therefore skin should be frozen to -80°C, whole, as soon as available. S9 should only be generated on

the day of the metabolic assay, immediately on thawing a skin sample. Freeze-thawing of S9 should be avoided.

Immediate addition of cofactors to S9 benefited the metabolic potential of the enzymes involved, which is in accordance with previous observations made on N-acetyltransferase activity (Binkley et al., 1976). In our study, GSH conjugation, methylation, N-acetylation, sulphation and glucuronidation all took place to some extent thanks to the addition of the cofactors. This is in accordance with general procedures for metabolic studies using liver fractions (Iyer and Sinz, 1999). Historically, extensive work has been carried out to characterise the biosynthesis pathways controlling cofactors ability in liver (Reinke L.A., 1994). It is not clear whether skin cells carry out the totality of synthesis of cofactors necessary to Phase II metabolism or whether some cofactors can be imported from other body organs via plasma. Although it is difficult to pinpoint which foods could be specifically beneficial for the induction of xenobiotic metabolising enzymes in skin by increasing the availability of cofactors, nutrition might be a differentiating factor in healthy donors. The consumption of certain foods such as cruciferous has been linked to an increase of metabolic activity for UGTs and GSTs. Isolated active ingredients such as resveratrol or quercetin, available in certain foods, have also increased metabolic activity in vivo (Hodges and Minich, 2015).

However, a Phase II metabolism study carried out using freshly excised skin maintained in medium and used immediately (Manevski et al., 2015) did not benefit from the addition of cofactors to the medium and the clearance rates of the chemical probes tested remained unchanged. The differences observed between a study using homogenised skin S9 and whole skin in culture highlight the importance of the localisation of the enzymes themselves. Human skin has a complex structure and is constituted of many cell layers, at different stages of differentiation. For metabolism study purposes, three distinct layers namely the *stratum corneum* (cornocytes in a lipid rich layer), the epidermis (keratinocytes) and the dermis (fibroblasts in a collagen matrix) can be of interest. Studies carried out in skin explants by Lu-The et al showed that the epidermis contained up to ten times more mRNA for SULT2B1b than the dermis, while SULT1A1 mRNA was found primarily in the dermis (Luu-The et al., 2009). While GST activity in human

skin as well as rat skin was also thought to be more prominent in the dermis, GST had been localized primarily in the sebaceous glands and outer root sheath of hair follicles of rat skin (Raza et al., 1991). Another study located UGT activity in the *stratum corneum* (Peters et al., 1987). The use of S9 allows to investigate all these enzymes in a single homogenate and was our preferred approach for this study.

Measuring rates of metabolism in skin is key to understanding the bioavailability of topically applied chemicals, both systemically and locally (Gibbs et al., 2007). Therefore, any protocol chosen needs to be standardised to enable direct comparison of results. One must be aware when interpreting the data shown here that due to the artificially optimal conditions used (i.e. excess of cofactors) the data might not be fully representative of the scenario of clearance *in vivo*.

The availability of the cofactor, for example i.e. the capacity of skin to generate or import cofactors on demand, could also play an important part in the defence system against exogenous chemicals. As clearly illustrated by comparing rates of glutathione conjugation with and without the addition of GSH (Supplementary data Figure C), cofactor amounts are an important influence on metabolic rates (Spriggs et al., 2016) and should be considered when studying inter-individual variability using sub cellular fractions. In addition, it was also found in the initial method development stages, that co-incubating all cofactors and drugs simultaneously induced a competition of enzymes for substrate and/or cofactors resulting in an inhibitory effect on one another compared to when incubated separately; this was particularly the case when a test chemical could undergo multiple metabolic pathways (Data not shown). This has been shown previously in the case of inhibition of NAT-1 in keratinocytes by competing substrates (Kawakubo et al., 1990).

Using our optimised and standardised assay, we analysed skin samples from 90 donors. The first observation we made was that there did not seem to be any statistical differences between gender, and age played a very limited role in enzymatic levels observed. Only GST activity was shown to decrease with the age of the donors, which is not surprising as GSH production tends to decrease with ageing (Maher, 2005) and GST activity is closely linked to the availability of its cofactor. The variability in the physiology and appearance of skin has also be evaluated against factors related to lifestyle. For example, a study

reported that the thickness of the *stratum corneum* could be negatively correlated to the number of years the donors had been smoking (Sandby-Moller et al., 2003). Conversely, moderate protective effects against UV can be observed in volunteers supplemented with vitamins E and C, carotenoids and polyunsaturated fatty acids in various combinations on top of their usual diet (Boelsma et al., 2001). It is possible that the same factors, which we could not investigate specifically here, might influence metabolic capacity.

We tried to identify any donor whose metabolic activities were clearly in the top or bottom 25% of the values recorded. While the donors presenting an overall low metabolic activity could be real “low metabolisers”, it is likely that the sample handling procedure post-surgery might also have played a role in decreasing the viability of these five specific skin samples. On the other hand, donors with high overall metabolic capacity had individual rates that were different enough from the other members of this group to limit the likelihood that the results were due to sample handling alone. Lacking the information from medical conditions, including skin allergy, that these donors might have, it is difficult to link the high metabolic activity to either lifestyle, health effects or induction due to the regular prescription of medical drugs. It has been hypothesised that some individuals have a metabolic predisposition that leads to skin reactions when dermally applying sulphonamides (Wolkenstein et al., 1995) but this has not been fully explored in the area of skin allergy. There is now a potential to explore in future studies whether patients with known allergies possess a different metabolic capacity to the general population using the data presented here.

### Conclusion

The use of subcellular fractions derived from skin, such as S9, is a practical approach for comparing inter-individual enzyme activity when many skin samples are available, providing the protocols for excision of the skin and handling in the laboratory are carefully controlled. S9 samples can be of use in predicting skin metabolism as part of a PBPK model and the approach is akin to the use of subcellular fractions in predicting liver metabolism (Harrison et al., 2012) although an analogous scaling approach is yet to be devised for skin. We presented a standardised protocol for the generation of skin metabolism data using



S9, which can easily be used for any specific probe of interest in the future. Applying this methodology to a 90-donor study, has enabled us to define the metabolic rates for Phase II enzymes in a format allowing the introduction of these data into mathematical models to refine the biological understanding of human skin and its role as a detoxifying organ. This might help increasing the mechanistic relevance of risk assessments and reducing uncertainty linked to chemical fate and systemic exposure.

## References

- Alotto, D., Ariotti, S., Graziano, S., Verrua, R., Stella, M., Magliacani, G., Castagnoli, C., 2002. The role of quality control in a skin bank: tissue viability determination. *Cell Tissue Bank* 3, 3-10.
- Berard, F., Marty, J.P., Nicolas, J.F., 2003. Allergen penetration through the skin. *Eur J Dermatol* 13, 324-330.
- Berglund, S.R., Schwieter, C.W., Jones, A.A., Stern, R.L., Lehmann, J., Goldberg, Z., 2007. Optimized methodology for sequential extraction of RNA and protein from small human skin biopsies. *J Invest Dermatol* 127, 349-353.
- Binkley, S., Klein, D.C., Weller, J.L., 1976. Pineal serotonin N-acetyltransferase activity: protection of stimulated activity by acetyl-CoA and related compounds. *J Neurochem* 26, 51-55.
- Boelsma, E., Hendriks, H.F., Roza, L., 2001. Nutritional skin care: health effects of micronutrients and fatty acids. *Am J Clin Nutr* 73, 853-864.
- Bonifas, J., Hennen, J., Dierolf, D., Kalmes, M., Blömeke, B., 2010. Evaluation of cytochrome P450 1 (CYP1) and n-acetyltransferase 1 (NAT 1) activities in HaCaT cells: Implications for the development of in vitro techniques for predictive testing of contact sensitizers. *Toxicology in vitro* 24, 973-980.
- Bouwstra, J.A., Ponc, M., 2006. The skin barrier in healthy and diseased state. *Biochim Biophys Acta* 1758, 2080-2095.
- Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254.
- Castagnoli, C., Alotto, D., Cambieri, I., Casimiri, R., Aluffi, M., Stella, M., Alasia, S.T., Magliacani, G., 2003. Evaluation of donor skin viability: fresh and cryopreserved skin using tetrazolium salt assay. *Burns* 29, 759-767.
- de Wever, B., Kurdykowski, S., Descargues, P., 2015. Human Skin Models for Research Applications in Pharmacology and Toxicology: Introducing NativeSkin®, the “Missing Link” Bridging Cell Culture and/or Reconstructed Skin Models and Human Clinical Testing. *Applied In Vitro Toxicology* 1, 26-32.
- Dreyfuss, J., Bigger, J.T., Jr., Cohen, A.I., Schreiber, E.C., 1972. Metabolism of procainamide in rhesus monkey and man. *Clin Pharmacol Ther* 13, 366-371.
- Eilstein, J., Lereaux, G., Budimir, N., Hussler, G., Wilkinson, S., Duche, D., 2014. Comparison of xenobiotic metabolizing enzyme activities in ex vivo human skin and reconstructed human skin models from SkinEthic. *Arch Toxicol* 88, 1681-1694.

- Gajewska, M., Paini, A., Sala Benito, J.V., Burton, J., Worth, A., Urani, C., Briesen, H., Schramm, K.W., 2015. In vitro-to-in vivo correlation of the skin penetration, liver clearance and hepatotoxicity of caffeine. *Food Chem Toxicol* 75, 39-49.
- Gibbs, S., van de Sandt, J.J., Merk, H.F., Lockley, D.J., Pendlington, R.U., Pease, C.K., 2007. Xenobiotic metabolism in human skin and 3D human skin reconstructs: a review. *Curr Drug Metab* 8, 758-772.
- Goebel, C., Hewitt, N.J., Kunze, C., Wenker, W., Hein, D.W., Beck, H., Skare, J., 2009. Skin metabolism of aminophenols: human keratinocytes as a suitable in vitro model to qualitatively predict the dermal transformation of 4-amino-2-hydroxytoluene in vivo. *Toxicology and Applied Pharmacology* 235, 114-123.
- Gotz, C., Pfeiffer, R., Tigges, J., Blatz, V., Jackh, C., Freytag, E.M., Fabian, E., Landsiedel, R., Merk, H.F., Krutmann, J., Edwards, R.J., Pease, C., Goebel, C., Hewitt, N., Fritsche, E., 2012a. Xenobiotic metabolism capacities of human skin in comparison with a 3D epidermis model and keratinocyte-based cell culture as in vitro alternatives for chemical testing: activating enzymes (Phase I). *Exp Dermatol* 21, 358-363.
- Gotz, C., Pfeiffer, R., Tigges, J., Ruwiedel, K., Hubenthal, U., Merk, H.F., Krutmann, J., Edwards, R.J., Abel, J., Pease, C., Goebel, C., Hewitt, N., Fritsche, E., 2012b. Xenobiotic metabolism capacities of human skin in comparison with a 3D-epidermis model and keratinocyte-based cell culture as in vitro alternatives for chemical testing: phase II enzymes. *Exp Dermatol* 21, 364-369.
- Harris, I.R., Siefken, W., Beck-Oldach, K., Brandt, M., Wittern, K.P., Pollet, D., 2002. Comparison of activities dependent on glutathione S-transferase and cytochrome P-450 IA1 in cultured keratinocytes and reconstructed epidermal models. *Skin Pharmacol Appl Skin Physiol* 15 Suppl 1, 59-67.
- Harrison, A., Gardner, I., Hay, T., Dickins, M., Beaumont, K., Phipps, A., Purkins, L., Allan, G., Christian, R., Duckworth, J., 2012. Case studies addressing human pharmacokinetic uncertainty using a combination of pharmacokinetic simulation and alternative first in human paradigms. *Xenobiotica* 42, 57-74.
- Hewitt, N.J., Edwards, R.J., Fritsche, E., Goebel, C., Aeby, P., Scheel, J., Reisinger, K., Ouédraogo, G., Duche, D., Eilstein, J., Latil, A., Kenny, J., Moore, C., Kuehn, J., Barroso, J., Fautz, R., Pfuhler, S., 2013. Use of Human In Vitro Skin Models for Accurate and Ethical Risk Assessment: Metabolic Considerations. *Toxicological Sciences* 133, 209-217.
- Hodges, R.E., Minich, D.M., 2015. Modulation of Metabolic Detoxification Pathways Using Foods and Food-Derived Components: A Scientific Review with Clinical Application. *J Nutr Metab* 2015, 760689.
- Hu, T., Khambatta, Z.S., Hayden, P.J., Bolmarcich, J., Binder, R.L., Robinson, M.K., Carr, G.J., Tiesman, J.P., Jarrold, B.B., Osborne, R., Reichling, T.D., Nemeth, S.T., Aardema, M.J., 2010. Xenobiotic metabolism gene expression in the EpiDerm in vitro 3D human epidermis model compared to human skin. *Toxicol In Vitro* 24, 1450-1463.
- Iyer, K.R., Sinz, M.W., 1999. Characterization of Phase I and Phase II hepatic drug metabolism activities in a panel of human liver preparations. *Chem Biol Interact* 118, 151-169.
- Jackh, C., Blatz, V., Fabian, E., Guth, K., van Ravenzwaay, B., Reisinger, K., Landsiedel, R., 2011. Characterization of enzyme activities of Cytochrome P450 enzymes, Flavin-dependent monooxygenases, N-acetyltransferases and UDP-glucuronyltransferases in human reconstructed epidermis and full-thickness skin models. *Toxicol In Vitro* 25, 1209-1214.
- Jacques, C., Perdu, E., Duplan, H., Jamin, E.L., Canlet, C., Debrauwer, L., Cravedi, J.P., Mavon, A., Zalko, D., 2010. Disposition and biotransformation of 14C-Benzo(a)pyrene in a pig ear skin model: ex vivo and in vitro approaches. *Toxicol Lett* 199, 22-33.
- Jacques, C., Perdu, E., Jamin, E.L., Cravedi, J.P., Mavon, A., Duplan, H., Zalko, D., 2014. Effect of skin metabolism on dermal delivery of testosterone: qualitative assessment using a new short-term skin model. *Skin Pharmacol Physiol* 27, 188.
- Kawakubo, Y., Yamazoe, Y., Kato, R., Nishikawa, T., 1990. High capacity of human skin for N-acetylation of arylamines. *Skin Pharmacol* 3, 180-185.
- Korkina, L., 2016. Metabolic and redox barriers in the skin exposed to drugs and xenobiotics. *Expert Opin Drug Metab Toxicol* 12, 377-388.
- Lebonvallet, N., Jeanmaire, C., Danoux, L., Sibille, P., Pauly, G., Misery, L., 2010. The evolution and use of skin explants: potential and limitations for dermatological research. *Eur J Dermatol* 20, 671-684.

- Luu-The, V., Duche, D., Ferraris, C., Meunier, J.R., Leclaire, J., Labrie, F., 2009. Expression profiles of phases 1 and 2 metabolizing enzymes in human skin and the reconstructed skin models Episkin and full thickness model from Episkin. *J Steroid Biochem Mol Biol* 116, 178-186.
- Madison, K.C., 2003. Barrier function of the skin: "la raison d'etre" of the epidermis. *J Invest Dermatol* 121, 231-241.
- Maher, P., 2005. The effects of stress and aging on glutathione metabolism. *Ageing Res Rev* 4, 288-314.
- Maier, T., Guell, M., Serrano, L., 2009. Correlation of mRNA and protein in complex biological samples. *FEBS Lett* 583, 3966-3973.
- Manevski, N., Swart, P., Balavenkatraman, K.K., Bertschi, B., Camenisch, G., Kretz, O., Schiller, H., Walles, M., Ling, B., Wettstein, R., Schaefer, D.J., Itin, P., Ashton-Chess, J., Pognan, F., Wolf, A., Litherland, K., 2015. Phase II metabolism in human skin: skin explants show full coverage for glucuronidation, sulfation, N-acetylation, catechol methylation, and glutathione conjugation. *Drug Metab Dispos* 43, 126-139.
- Mannisto, P.T., Kaakkola, S., 1999. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. *Pharmacol Rev* 51, 593-628.
- Manwaring, J., Rothe, H., Obringer, C., Foltz, D.J., Baker, T.R., Troutman, J.A., Hewitt, N.J., Goebel, C., 2015. Extrapolation of systemic bioavailability assessing skin absorption and epidermal and hepatic metabolism of aromatic amine hair dyes in vitro. *Toxicol Appl Pharmacol* 287, 139-148.
- Møllgaard, B., Hoelgaard, A., Bundgaard, H., 1982. Pro-drugs as drug delivery systems XXIII. Improved dermal delivery of 5-fluorouracil through human skin via N-acyloxymethyl pro-drug derivatives. *International Journal of Pharmaceutics* 12, 153-162.
- Oesch, F., Fabian, E., Guth, K., Landsiedel, R., 2014. Xenobiotic-metabolizing enzymes in the skin of rat, mouse, pig, guinea pig, man, and in human skin models. *Arch Toxicol* 88, 2135-2190.
- Peters, W.H., Allebes, W.A., Jansen, P.L., Poels, L.G., Capel, P.J., 1987. Characterization and tissue specificity of a monoclonal antibody against human uridine 5'-diphosphate-glucuronosyltransferase. *Gastroenterology* 93, 162-169.
- Raza, H., Awasthi, Y.C., Zaim, M.T., Eckert, R.L., Mukhtar, H., 1991. Glutathione S-transferases in human and rodent skin: multiple forms and species-specific expression. *J Invest Dermatol* 96, 463-467.
- Reinke L.A., K.F.C., and Thurman R.G 1994. Cofactor Supply as a Rate-Limiting Determinant of Hepatic Conjugation Reactions Conjugation-Deconjugation Reactions in Drug Metabolism and Toxicity, ed Kauffman FC (Springer-Verlag, Berlin), 219-244.
- Sandby-Møller, J., Poulsen, T., Wulf, H.C., 2003. Epidermal thickness at different body sites: relationship to age, gender, pigmentation, blood content, skin type and smoking habits. *Acta Derm Venereol* 83, 410-413.
- Schmidt, R.J., Khan, L., 1989. Evidence for the Existence of Prohaptan-Activating Enzyme in the Skin: Induction of Contact Sensitivity to Monocrotaline in Mice. In: Frosch, P., Dooms-Goossens, A., Lachapelle, J.M., Rycroft, R.J.G., Scheper, R.J. (Eds.) *Current Topics in Contact Dermatitis*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 24-30.
- Spriggs, S., Sheffield, D., Olayanju, A., Kitteringham, N.R., Naisbitt, D.J., Aleksic, M., 2016. Effect of Repeated Daily Dosing with 2,4-Dinitrochlorobenzene on Glutathione Biosynthesis and Nrf2 Activation in Reconstructed Human Epidermis. *Toxicol Sci* 154, 5-15.
- Svensson, C.K., 2009. Biotransformation of Drugs in Human Skin. *Drug Metabolism and Disposition* 37, 247-253.
- van Eijl, S., Zhu, Z., Cupitt, J., Gierula, M., Gotz, C., Fritsche, E., Edwards, R.J., 2012. Elucidation of xenobiotic metabolism pathways in human skin and human skin models by proteomic profiling. *PLoS One* 7, e41721.
- Varani, J., Perone, P., Spahlinger, D.M., Singer, L.M., Diegel, K.L., Bobrowski, W.F., Dunstan, R., 2007. Human skin in organ culture and human skin cells (keratinocytes and fibroblasts) in monolayer culture for assessment of chemically induced skin damage. *Toxicol Pathol* 35, 693-701.
- Wang, Q., Jia, R., Ye, C., Garcia, M., Li, J., Hidalgo, I.J., 2005. Glucuronidation and sulfation of 7-hydroxycoumarin in liver matrices from human, dog, monkey, rat, and mouse. *In Vitro Cell Dev Biol Anim* 41, 97-103.

Wiegand, C., Hewitt, N.J., Merk, H.F., Reisinger, K., 2014. Dermal xenobiotic metabolism: a comparison between native human skin, four in vitro skin test systems and a liver system. *Skin Pharmacol Physiol* 27, 263-275.

Wolkenstein, P., Charue, D., Laurent, P., Revuz, J., Roujeau, J.C., Bagot, M., 1995. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. *Arch Dermatol* 131, 544-551.

Zalko, D., Jacques, C., Duplan, H., Bruel, S., Perdu, E., 2011. Viable skin efficiently absorbs and metabolizes bisphenol A. *Chemosphere* 82, 424-430.